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Impact of *Morus alba* L. on the expression of drug-metabolizing enzymes and transporters in Caco-2 cells

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ABSTRACT

*Morus alba (*MA) L. (mulberry) fruit has been consumed for a long time as a food and a source of vitamin C, anthocyanins, flavonoids, and alkaloids. The effects of MA fruit extract on drug-metabolizing genes and drug transporters were investigated in human colorectal adenocarcinoma cells. Cells treated with MA or its major bioactive constituents, rutin, and cyanidin-3-O-glucoside (C3G), were assessed for cell viability, production of reactive oxygen species (ROS), and alanine aminotransferase (ALT) and aspartate transaminase (AST) levels. The mRNA expression of target metabolic genes was determined using real-time polymerase chain reaction. Cell viability remained higher than 80%, and ROS levels were unchanged by all treatments. MA, C3G, and rutin did not alter the ALT and AST levels or expression of *CYP2D6* and sulfotransferase 1A1 (*SULT1A1*). However, MA, C3G, and rutin down-regulated the expression of *CYP2C19*, *CYP3A4*, uridine diphosphate-glucuronosyltransferase 1A6 (*UGT1A6*), *N*-acetyltransferase 1, and organic anion transporting polypeptide 1B1 (*OATP1B1*), and MA elevated the expression of *CYP1A2*. In addition, MA induced *CYP1A2* and *CYP2C19* expression in combination with aspirin, caffeine, and simvastatin and upregulated expression of *UGT1A6* and *SULT1A1* in combination with paracetamol. Therefore, consumption of MA fruit or its supplements poses a risk for drug interactions via its modulation of cytochrome P450 and conjugation enzyme-associated metabolism and *OATP1B1*-mediated drug transport.

INTRODUCTION

The fruit of *Morus alba* (MA) L. (mulberry), from the family Moraceae, has been consumed as a food and dietary supplement for a long time and is rich in vitamin C, anthocyanins, flavonoids, and alkaloids. MA fruit and fruit extracts have shown antioxidant, antidiabetic, antiatherosclerotic, antiobesity, anticholesterol, and antibacterial activities, as well as immune enhancement, neuroprotective, and hepatoprotective properties (Chan *et al*., 2016; Chang *et al*., 2021; Hansawasdi and Kawabata, 2006; Zafar *et al*., 2013; Zhang *et al*., 2018). MA extracts are considered safe to use without severe side effects. MA leaf extract exhibited no acute toxicity in rats at doses above 5 g/kg (Abdulla *et al*., 2009), but mild itching was reported after topical application of MA oil for melasma treatment (Alvin *et al*., 2011). MA has been used as a nutraceutical product or food supplement due to the presence of several functional and pharmacological compounds in its leaves, fruit, and seeds, such as mulberroside A, rutin, chlorogenic acid, caffeic acid, quercetin, gallic acid, kaempferol, and apigenin (Chen *et al*., 2021; Hansawasdi and Kawabata, 2006; Mei *et al*., 2012).

All orally consumed substances have the potential for inter with medications (Fugh-Berman, 2000). Polypharmacy is an increasingly common event with physicians needing to prescribe multiple medications for patients with multiple conditions. In addition, people have access to a wide range of over-the-counter medications as well as health and dietary supplements. Herb-drug interactions may modulate the pharmacological and toxicological effects of either the herb or the drug. Information about MA herb-drug interactions is very limited.

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Cytochrome P450 (CYP) monooxygenase enzymes, such as *CYP1A2, CYP2C9*, and *CYP3A4*, are responsible for phase I metabolism of greater than half of all clinical drugs (Zanger and Schwab, 2013). Foods or supplements can affect the metabolism of clinical drugs by interfering with the regulatory expression of CYPs. Conjugation enzymes involved in hepatic phase II metabolism including uridine diphosphate- (UDP-) glucuronosyltransferase 1A6 (*UGT1A6*), *N*-acetyltransferase 1 (*NAT1*), and sulfotransferase 1A1 (*SULT1A1*) can also play an important role in the metabolism of a variety of clinical drugs, xenobiotics, and toxicants (Kim *et al*., 2019; Rasool *et al*., 2019; Witham *et al*., 2017). In addition to phaseaction I and II metabolizing enzymes, some drug transporters such as organic anion transporting polypeptide 1B1 (*OATP1B1*) and ATP-binding cassette (ABC) transporter protein 1 (*ABCB1* or P-glycoprotein) are important determinants for transporter-mediated drug interactions (Chen *et al*., 2019; Kayesh *et al*., 2021; Meszaros *et al*., 2013; Sorf *et al*., 2018).

A monolayer of human colorectal adenocarcinoma cells (Caco-2) is the most suitable *in vitro* model representing the human intestine for pharmacokinetic studies, especially for studies of the bioactivity of foods and herbal supplements (Brück *et al*., 2017; Iftikhar *et al*., 2020; Vaessen *et al*., 2017). The reduction of oxygen during normal biochemical or metabolic reactions in living cells or organisms results in the production of intermediate metabolic byproducts known as reactive oxygen species (ROS), which can cause oxidative damage to cells or tissues (Kükürt *et al*., 2021). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are enzyme markers of oxidative damage to hepatocytes but can also be released by other damaged cell types (Sepulveda, 2019; Udomsak *et al*., 2022). Hence, this study investigated the effects of MA on ROS, AST, ALT, and the mRNA expression of several important phases I and II drug-metabolizing enzymes and drug transporters in a Caco-2 monolayer model.

MATERIALS AND METHODS

Reagents

Cyanidin-3-O-glucoside (C3G) and rutin were supplied by Chengdu Alfa Biotechnology Co., Ltd. (Chengdu, China). High-performance liquid chromatography (HPLC) solvents were products of RCI Labscan Ltd. (Bangkok, Thailand). Dulbecco's modified Eagle medium (DMEM) (+) phenol red, DMEM/F12 phenol red-free medium, Dulbecco's phosphate-buffered saline, fetal bovine serum (FBS), 1× penicillin, streptomycin, and neomycin antibiotics (PSN), and 1× GlutaMAX® were supplied by Gibco® (Thermo Fisher Scientific, MA). Resazurin, 2,4-dinitrophenylhydrazine (DNPH), 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), rifampicin, and ketoconazole, *L*-aspartate, *L*-alanine, and α-ketoglutarate were products of Sigma-Aldrich (Missouri). ReverTra Ace®, random primers, and other reagents for reverse transcription were products of Toyobo Co., Ltd. (Osaka, Japan). Primers were synthesized by Bio Basic, Inc. (Markham, Ontario, CA). *Taq* polymerase, RNase inhibitor, deoxynucleotide triphosphate mixture, and other reagents for polymerase chain reaction (qPCR) were products of Vivantis Technologies Sdn. Bhd. (Selangor, Malaysia). Other laboratory chemicals were obtained from commercial suppliers with the highest purity and quality.

Preparation of MA fruit powder

Seedlings of MA L. cultivar Chiangmai were provided by the Queen Sirikit Department of Sericulture, Ministry of Agriculture and Cooperatives, Thailand, and were grown in Noen-Ruang Village, Muang District, Khon Kaen, Thailand. The fruits of MA were collected in March 2021. A plant specimen (Panya, T. 8 KKU No. 25978) was deposited at the Research Institute for Human High Performance and Health Promotion, Khon Kaen University. The MA fruits were washed in cold water three times and dried in a hot air oven at 50°C until the moisture content was 10%. The dried fruits were mashed and sieved (60 mesh) before storage in a vacuum aluminum package until further analysis.

HPLC analysis

MA fruit powder, C3G, and rutin were accurately weighed and dissolved in absolute methanol before being filtered using a 0.45 µm membrane. The C3G and rutin contents of MA fruit powder were analyzed by HPLC-UV. A Hypersil ODS (4.0 × 250 mm × 5 µm, Agilent Technologies Inc., Santa Clara, CA) column was used as the stationary phase. A gradient mobile phase of acetonitrile in 0.1% phosphoric acid was increased from 5% to 80% in 25 minutes with a flow rate of 1 ml/minute. The machine (Prominence-i Model LC-2030C 3D, Shimadzu Corporation, Kyoto, Japan) was set up with dual UV detectors at 517 nm for C3G and 365 nm for rutin. The HPLC method validation and the chromatogram are shown in Table 1 and Figure 1.

Cell culture

Caco-2 cells (RBRC-RBC0988) were supplied by the Cell Bank of RIKEN BioResource Center (Saitama, Japan). The cells were maintained in DMEM (1 g/l *D*-glucose) with 20% FBS, *L*-glutamine, sodium pyruvate, 1× GlutaMAX®, and 1× PSN at 37°C with 5% CO_2 and 95% relative humidity. The cells were cultured as a monolayer in 24-well plates (2.5×10^5 cells/well in 0.5 ml). At 48 hours after seeding, they were incubated with 0.1% (v/v) dimethyl sulfoxide (DMSO, control), 10 µM ketoconazole (Keto), 10 µM rifampicin (Rif), 10 µM simvastatin (Sim), 50 µM caffeine (Caf), 5 mM of paracetamol (APAP), 5 mM aspirin (ASA), 1–10 µM of C3G, 1–10 µM rutin, and/or 125–500 µg/ml of MA for 48 hours. The cells were harvested for quantitative analysis by real-time polymerase chain reaction (RT/qPCR) ($n = 4$–5 replicates per group with two independent experiments). In addition, the cell culture medium was collected for the determination of ROS, ALT, and aspartate transaminase (AST) levels.

Determination of cell viability

Cell viability was determined by the resazurin assay (Alamar Blue assay) as previously described (Sriset *et al*., 2021). Briefly, the living cells were incubated with 100 µM resazurin in 5% CO_2 at 37°C for 30 minutes. The resorufin formation was measured for spectrofluorometric intensity at excitation of 530 nm and emission of 580 nm. The percentage of cell viability was

Table 1. Reversed phase HPLC validation and rutin and C3G contents in MA extract.

	Rutin	C3G
Specificity		
λ_{max} (nm)	365	517
Retention time (minutes)	14.1	13.5
Linearity		
Concentration range (µg/ml)	20 - 120	20 - 120
Linear regression equation	$y = 27{,}698\times - 72{,}894$	$y = 36{,}529\times - 23{,}590$
R^2	0.9988	0.9992
Accuracy		
% Recovery ± % RSD	100.41 ± 1.09	100.28 ± 1.02
Precision (% RSD)		
Within day	0.29–1.47	0.28–1.34
Between day	0.56–1.34	0.63–1.82
Sensitivity		
LOD (µg/ml)	5.76	3.51
LOQ (µg/ml)	17.47	10.65
% Content in MA (± SD)	7.85 ± 0.29	7.65 ± 0.31

calculated from an increasing rate of resorufin compared to the nontreatment group.

Determination of ROS

The ROS level in the cell culture medium was measured using the DCFH-DA reaction as previously described (Sriset *et al*., 2021). The medium was incubated in the dark with 62.5 nM DCFH-DA for 40 minutes. The fluorescence intensity of 2′-7′dichlorofluorescein was measured at excitation and emission wavelengths of 484 and 530 nm, respectively, using a spectrofluorometer. The ROS level was calculated by comparison with a standard curve of hydrogen peroxide (2.5 to 20 µM).

Determination of cellular transaminases (AST and ALT)

An ALT substrate (300 mM *L*-alanine and 0.7 mM α-ketoglutarate) or AST substrate (10 mM *L*-aspartate and 1.7 mM α-ketoglutarate) was incubated with a cell culture medium or sodium pyruvate standard solution at 37C for 20–30 minutes. Then, 5 mM DNPH was added, and the mixture was left at room temperature for 20 minutes. The reaction was stopped by the addition of 1.3 N sodium hydroxide. The absorbance of the mixture was measured at 505 nm. The levels of AST and ALT were determined as international units per liter (IU/l) by comparison with a standard curve of sodium pyruvate (Sriset *et al*., 2021).

Quantitative determination of mRNA expression by reverse transcription (RT/qPCR)

At 48 hours after treatment, Caco-2 cells were collected using a guanidinium thiocyanate-phenol solution. Chloroform was added to extract RNA. The RNA was precipitated by isopropanol, and the pellet was washed twice with cold 70% ethanol before measuring the concentration, purity, and integrity of the RNA. Total RNA (500 ng per reaction) was reverse-transcribed using a ReverTra Ace® kit. The cDNA (20 ng per reaction) was used to determine the expression of target genes with specific primers (Table 2) using RT/qPCR with SYBR Green I probe detection. The expression of target genes was normalized to the reference gene, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The relative fold expression was calculated using the $^{\Delta\Delta}$Ct method.

Statistical analysis

All results are presented as mean ± standard deviation (SD) from 5 to 6 samples per group. Statistical differences were analyzed using one-way analysis of variances coupled with Tukey's procedure using IBM-SPSS version 23 software (Armonk, NY). $p \leq 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

The effect of MA fruit on cell viability, ROS, AST, and ALT

Phytochemical compounds including rutin, apigenin, chlorogenic acid, kaempferol, umbelliferone, morin, and luteolin have been isolated from the leaves, fruit, branches, bark, and roots of MA (Chu *et al*., 2006). Mulberroside A is a bioactive constituent previously found in the ethanolic extract of MA root (Park *et al*., 2011). In this study, we determined the mulberroside A, C3G, rutin, ellagic acid, chlorogenic acid, quercetin, kaempferol, galangin, genistein, luteolin, morin, myricetin, hesperidin, and silymarin contents of MA fruit powder, using HPLC (data not shown). The HPLC chromatogram (Fig. 1) indicates that rutin and C3G are the major constituents of MA. The rutin and C3G contents of MA were 7.85% ± 0.29% and 7.65% ± 0.31%, respectively (Table 1). These observations were consistent with a previous study that showed mulberry fruit contained a large amount of anthocyanins, which were mainly composed of cyanidin 3-rutinoside and C3G (Du *et al*., 2008). Rutin has been found in many parts of MA, including the leaves, fruit, and roots (Hunyadi *et al*., 2012; Zhao *et al*., 2015). The amount of these constituents can vary depending on the plant source, the harvest period, the method of extraction,

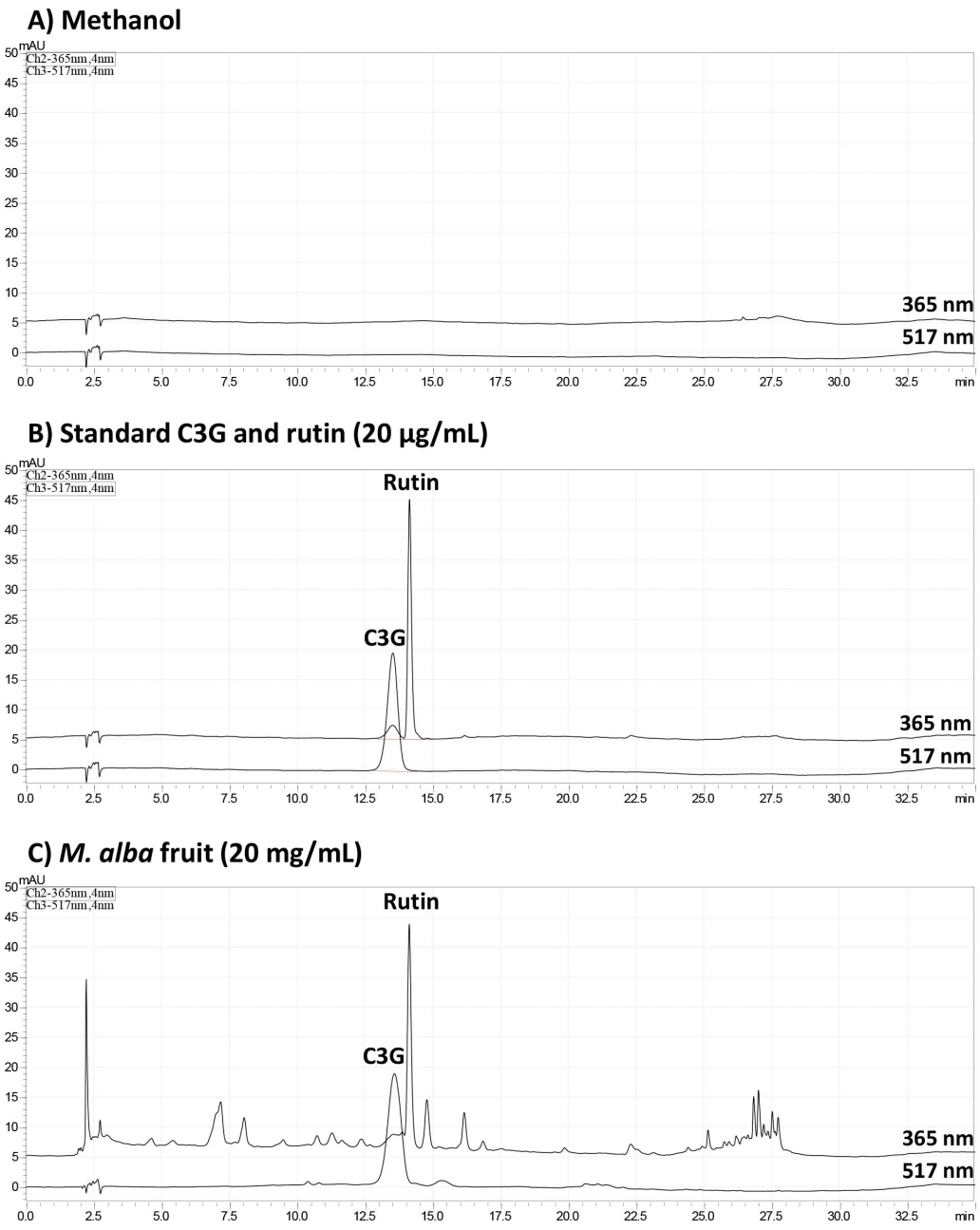

Figure 1. HPLC chromatogram of standard C3G and rutin and MA fruit.

the plant part used, and the method of analysis (Altemimi *et al*., 2017; Hussain *et al*., 2017). Since high levels of rutin and C3G were found in the MA sample, experiments in Caco-2 cells were continued with these two compounds. Previously, Zhang *et al*. (2013). examined the effect of 5 to 200 µM rutin on absorption and metabolism in Caco-2 cells (Zhang *et al*., 2013), and Ferrari *et al*. (2016) examined the anti-inflammatory and antioxidant activities of 20 to 40 µM C3G in Caco-2 cells (Ferrari *et al*., 2016). The doses of rutin and C3G (1 and 10 µM) and MA (125 to 500 µg/ml) employed in the current study were chosen to avoid cytotoxic effects and morphological changes to the Caco-2 cells while still determining their effects on the expression of metabolizing enzymes and transporters.

Although Rif, rutin (10 µM), and MA (125 µg/ml) significantly reduced cell viability (Fig. 2A), the cell viability remained above 80%, which enabled us to continue the experiments, especially as MA at higher doses (250 and 500 µg/ml) did not show toxicity. All treatments did not change the ROS level (Fig. 2B). Excessive ROS production occurs in tumor cells, including Caco-2 cells. Rutin (5–100 µM) has been shown to possess ROS scavenger activity (ben Sghaier et al., 2016), and C3G (20–40 µM) improved intracellular redox status through

Table 2. Specific primers for RT/qPCR.

Genes	Forward and reverse primers (5' to 3')	Product size (bp)	Annealing temp (°C)
CYP1A2	*F:* ACAAGGGACACAACGCTGAA *R:* AGGGCTTGTTAATGGCAGTG	160	60.0
CYP2C19	*F:* GGATTGTAAGCACCCCCTG *R:* TAAAGTCCCGAGGGTTGTTG	174	60.0
CYP2D6	*F:* AGCTTTCTGGTGACCCCATC *R:* GGACCCGAGTTGGAACTACC	135	61.1
CYP3A4	*F:* CTTCATCCAATGGACTGCATAAA *R:* TCCCAAGTATAACACTCTACACACACA	87	55.0
UGT1A6	*F:* AGCCCAGACCCTGTGTCCTA *R:* CCACTCGTTGGGAAAAAGTCA	76	58.2
NAT1	*F:* GAATTCAAGCCAGGAAGAAGCA *R:* TCCAAGTCCAATTTGTTCCTAGACT	151	60.0
SULT1A1	*F:* GTCACCGAGCTCCCATCTTC *R:* GTCTCCATCCCTGAGGGAATC	76	60.0
OATP1B1	*F:* GAATGCCCAAGAGATGATGCTT *R:* ACCCAGTGCAAGTGATTTCAAT	154	60.0
ABCB1	*F:* GGGATGGTCAGTGTTGATGGA *R:* GCTATCGTGGTGGCAAACAATA	110	60.0
GAPDH	*F:* CACCATCTTCCAGGAGCGAG *R:* GACTCCACGACGTACTCAGC	72	61.1

inhibition of NF-κB signaling (Ferrari *et al*., 2016), in Caco-2 cells. Moreover, phytochemical-rich MA has been shown to have antioxidant and anti-inflammatory capacities (Gryn-Rynko *et al*., 2016). Cellular transaminases (ALT and AST) are common markers of cell injury (Pandurangan and Kim, 2015; Sriset *et al*., 2021). Keto and Rif markedly elevated levels of AST (Fig. 2C) and ALT (Fig. 2D) in Caco-2 cells, while all other treatments did not. Keto and Rif (at 10 μM) have also been shown to modify the expression of CYPs and transporters in Caco-2 cells (Netsch *et al*., 2006; Takano *et al*., 1998). As MA did not show toxicity in Caco-2 cells, all tested compounds were employed using these concentrations.

Alteration of phase I CYP expression by MA fruit

CYP1A2 is constitutively expressed in the human liver and is detectable in other tissues and cell lines such as Caco-2 cells (Netsch *et al*., 2006; Zanger and Schwab, 2013). *CYP1A2* is responsible for the metabolism of antidepressants and antipsychotics, as well as anti-inflammatory, anesthetic, and analgesic drugs (Zhou *et al*., 2010). Keto extensively induced expression of *CYP1A2* mRNA ($p < 0.001$), while Rif, C3G, and rutin did not (Fig. 3A). All doses of MA markedly elevated *CYP1A2* expression.

CYP2C19 is responsible for the metabolism of proton pump inhibitors (e.g., omeprazole, esomeprazole, and pantoprazole), antidepressants (e.g., imipramine, amitriptyline, sertraline, and fluoxetine), hypnotics and sedatives (e.g., diazepam, clobazam, and phenobarbital), phenytoin, proguanil, nelfinavir, voriconazole, and clopidogrel (Uppugunduri *et al*., 2012). *CYP2C19* is inducible by several drugs such as rifampicin, dexamethasone, and phenobarbital (Rana *et al*., 2010). Rif significantly induced expression of *CYP2C19* mRNA, while Keto, C3G, rutin, and MA strongly suppressed *CYP2C19* (Fig. 3B). Rif has previously been shown to increase *CYP2C19* activity in human enterocytes (Glaeser *et al*., 2005). On the other hand, Keto has been reported as a *CYP2C19* inhibitor (Uppugunduri *et al*., 2012).

CYP2D6 plays an important role in the liver and brain with reg to the metabolism of drugs such as beta-blockers, opiates, antiarrhythmic agents, neuroleptics, and antidepressants as well as neurotransmitters (Darney *et al*., 2021). The expression of *CYP2D6* mRNA was unchanged by any of the treatments (Fig. 3C).

The most important metabolizing enzyme in phase I is *CYP3A4* which metabolizes a large and diverse range of molecules covering more than half of the clinical drugs, including antihypertensive, antibacterial, antifungal, and antiviral drugs, bronchodilators, and lipid-lowering agents (Zanger and Schwab, 2013). Rif significantly increased the expression of *CYP3A4* mRNA, while Keto, C3G, and MA suppressed *CYP3A4* expression. Rutin did not affect *CYP3A4* expression (Fig. 3D).

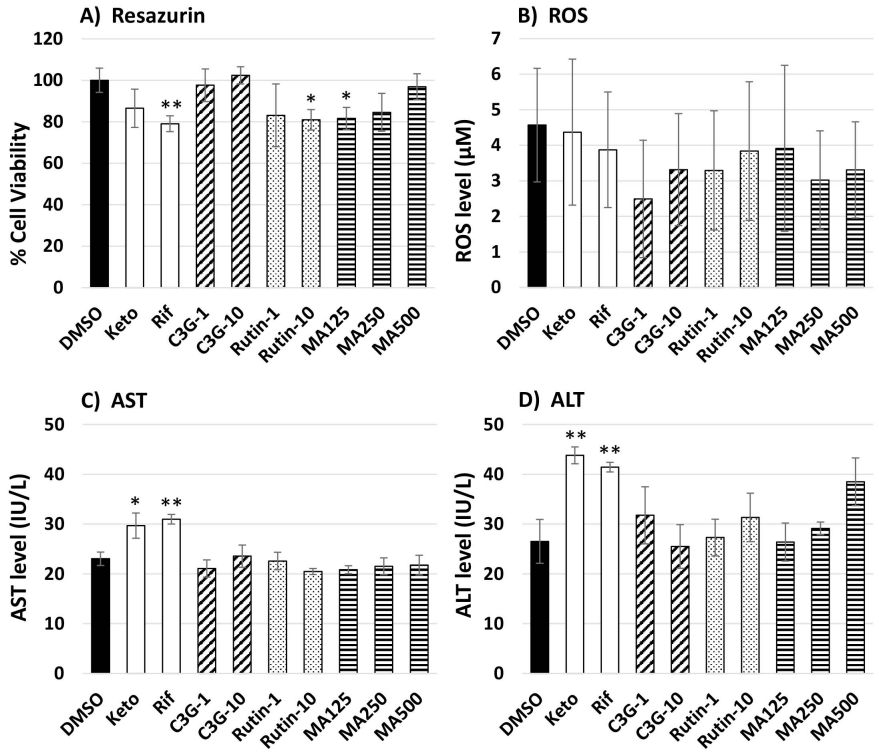

Figure 2. Effects of MA fruit, C3G , and rutin on cell viability, ROS, and cellular transaminases (AST and ALT) levels in Caco-2 cells. $^{*}p < 0.05$, $^{**}p < 0.001$ versus control (DMSO). Keto, 10 µM ketoconazole; Rif, 10 µM rifampicin; C3G, 1 and 10 µM C3G ; Rutin, 1 and 10 µM rutin; MA, 125, 250, and 500 µg/ml MA fruit (n = 4–5).

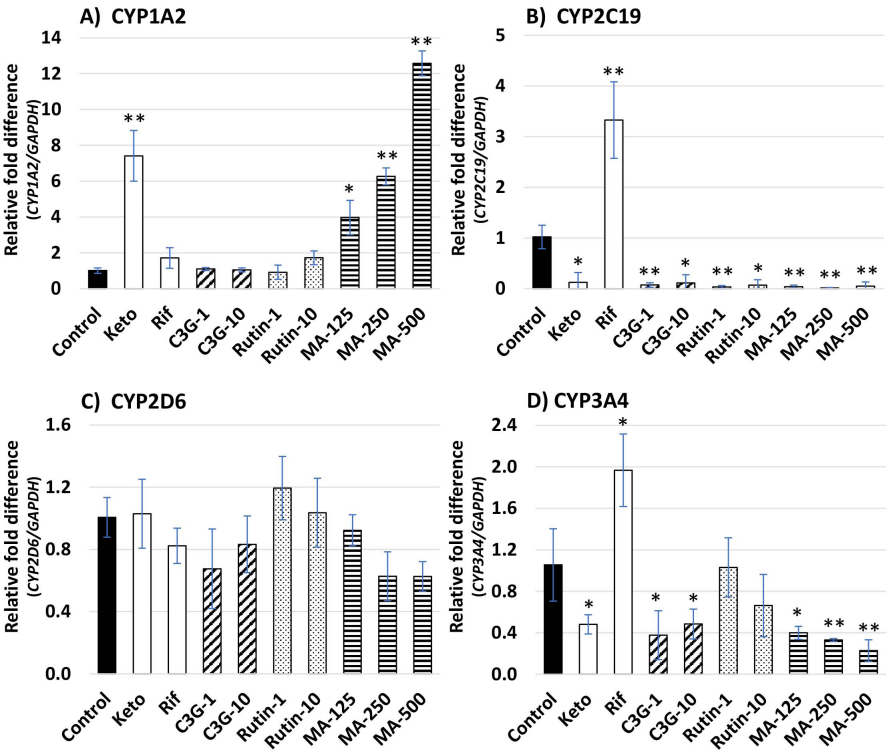

Figure 3. Effects of MA fruit, C3G , and rutin on CYP mRNA expression in Caco-2 cells. $^{*}p < 0.05$, $^{**}p < 0.001$ versus control (DMSO). Keto, 10 µM ketoconazole; Rif, 10 µM rifampicin; C3G, 1 and 10 µM C3G ; Rutin, 1 and 10 µM rutin; MA, 125, 250, and 500 µg/ml MA fruit (n = 4–5).

Keto has been proposed to induce *CYP1A2* via activation of the aryl hydrocarbon receptor and pregnane X receptor (PXR) (Novotna *et al*., 2014). Moreover, Keto was reported as an inhibitor for *CYP2C19* and transporters (Nikulin *et al*., 2017), while Rif was noted as a typical *CYP3A4* inducer (Kuncharoenwirat *et al*., 2020). Only a few studies have examined the effect of MA and its constituents on the expression of CYPs. However, anthocyanins (including C3G) have been shown to inhibit *CYP3A4* enzyme activity in human liver microsomes (Srovnalova *et al*., 2014), and an *in silico* analysis showed that rutin (quercetin-3-*O*-rutinoside) is metabolized by *CYP1A2* and *CYP2C9* (Sousa *et al*., 2013). More recently, Kar *et al.* (2015) performed an *in vitro* study focusing on the effect of different parts of MA on human recombinant CYP enzymes. While this approach has limitations for the study of induction, MA leaf extract inhibited the activities of human recombinant *CYP3A4*, *CYP2D6*, *CYP2C9*, and *CYP1A2* (Kar *et al*., 2015), which corresponds to the present findings for *CYP3A4* and *CYP2C19*. Therefore, potential MA-drug interactions could occur via *CYP1A2* induction and/or *CYP2C9* and *CYP3A4* suppression.

Alteration of phase II conjugation enzyme and transporter expression by MA fruit

The conjugation reactions of phase II metabolism are also keys to the biotransformation of drugs. *UGT1A6* is responsible for the biotransformation of a variety of drugs such as aspirin, carvedilol, naproxen, valproic acid, and zidovudine (Kim *et al*., 2019). In the present study, Rif extensively induced expression of *UGT1A6* mRNA, while Keto did not (Fig. 4A). In contrast, C3G, rutin, and MA significantly suppressed *UGT1A6* expression. These results correspond with previous observations that Rif induced *UGT1A6* expression in the human colon (van de Kerkhof *et al*., 2008), and morusin, a prenylated flavonoid isolated from the root bark of MA, inhibited expression of *UGT1A6, UGT1A7*, and *UGT1A8* in human, rat, dog, monkey, and minipig liver microsomes (Shi *et al*., 2016). Hence, MA and its constituents (C3G and rutin) possess the potential to downregulate *UGT1A6* expression in Caco-2 cells.

NAT1 is responsible for the metabolism of nitrogenous compounds such as aromatic amines/amides, hydrazine, and hydrazides and is also involved in the biotransformation, clearance, and toxicity of numerous pharmacologic agents and environmental toxicants (Walker *et al*., 2009). Rif, C3G, and MA suppressed expression of *NAT1,* while Keto and rutin did not show any significant change (Fig. 4B). Information about the effect of MA, C3G, and rutin on *NAT1* is limited, and this is the first report of the inhibitory effects of MA and C3G on *NAT1* expression in Caco-2 cells.

SULT1A1 is the key enzyme responsible for the sulfation of xenobiotics, including paracetamol, estrogens, and iodothyronines (Gamage *et al*., 2005; Rasool *et al*., 2019). Only Rif significantly induced the expression of *SULT1A1*, while the other tested compounds did not affect *SULT1A1* expression (Fig. 4C). Previously, the expression of *SULT2A1* mRNA was shown to be up-regulated by rifampicin in human hepatocytes (Fang *et al*., 2007). This might correlate with the observation that Rif induced *SULT1A1* expression in Caco-2 cells.

OATP1B1 has been reported to be associated with the uptake transport of clinical drugs, including statins, antibiotics, and anticancer drugs (Zhang *et al*., 2007). Keto, Rif, C3G, and rutin at 10 µM and all doses of MA significantly suppressed the expression of *OATP1B1* (Fig. 4D). The downregulation of *OATP1B1* expression by Keto and Rif in the present study confirms that these two compounds are inhibitors for OATPs (Choi *et al*., 2011; Pahwa *et al*., 2017). Similarly, flavonoids, quercetin, and rutin have previously been reported to inhibit the uptake of *OATP1B1* substrates (Wang *et al*., 2005).

ABCB1, the efflux transporter, also known as P-glycoprotein, regulates the plasma and intracellular concentrations of numerous xenobiotics (Gow *et al*., 2008). Keto, Rif, and C3G all slightly lowered the expression of *ABCB1* (although this was not statistically significant), and rutin did not affect *ABCB1* expression. The two higher tested doses of MA (250 and 500 µg/ml) suppressed *ABCB1* expression (Fig. 4E). This was consistent with a previous study that showed MA root extract suppressed *ABCB1* expression in human breast cancer MCF7 cells (Choi *et al*., 2013).

In summary, MA downregulated the expression of *UGT1A6*, *NAT1*, *OATP1B1*, and *ABCB1*. Therefore, MA fruit might cause drug interactions by disrupting of phase II biotransformation and drug transport via those related genes.

Effect of combinations of MA fruit and common drugs on phase I CYP expression

People are exposed to many different substances in their daily life that can affect phase I and II drug-metabolizing enzymes and transporters, especially through the consumption of commonly prescribed and over-the-counter medications. Paracetamol (acetaminophen or APAP) is a commonly used analgesic and antipyretic drug that is available in drugstores as an over-the-counter medication. APAP is mainly metabolized by *CYP2E1*, *CYP1A2*, and *CYP3A4*, and a metabolite of APAP, N-acetyl-*p*-benzoquinoneimine, can cause hepatotoxicity (Toes *et al*., 2005). Caffeine (Caf) is a bioactive component found in coffee, tea, soft drinks, and energy drinks. Although the daily intake of a cup of coffee, which contains 400 mg of Caf, is safe for health, caffeine can cause significant clinical pharmacokinetic interactions with many drugs (Belayneh and Molla, 2020). Low-dose aspirin (ASA) is recommended for the prevention of atherothrombotic and cardiovascular events. However, the antiplatelet action of ASA-induced drug interactions has high clinical importance (Saxena *et al*., 2013). Simvastatin (Sim) is a first-line antilipidemic drug commonly prescribed to reduce the risk of cardiovascular events, but it can also cause a variety of serious adverse effects such as myalgia, myopathy, and hepatotoxicity. Several drugs and compounds have been reported to disturb the metabolism of Sim, leading to an increase in the risk of these adverse effects (Kellick *et al*., 2014). Therefore, we examined the effect of MA combined with four commonly encountered compounds, APAP, ASA, Caf, and Sim, on profiles of CYPs and related genes in Caco-2 cells. On their own, APAP, ASA, Caf, and Sim did not change the expression of *CYP1A2* or *CYP2C19* mRNA, but when combined with MA, each of ASA, Caf, and Sim synergistically increased the expression of *CYP1A2* and *CYP2C19* mRNA

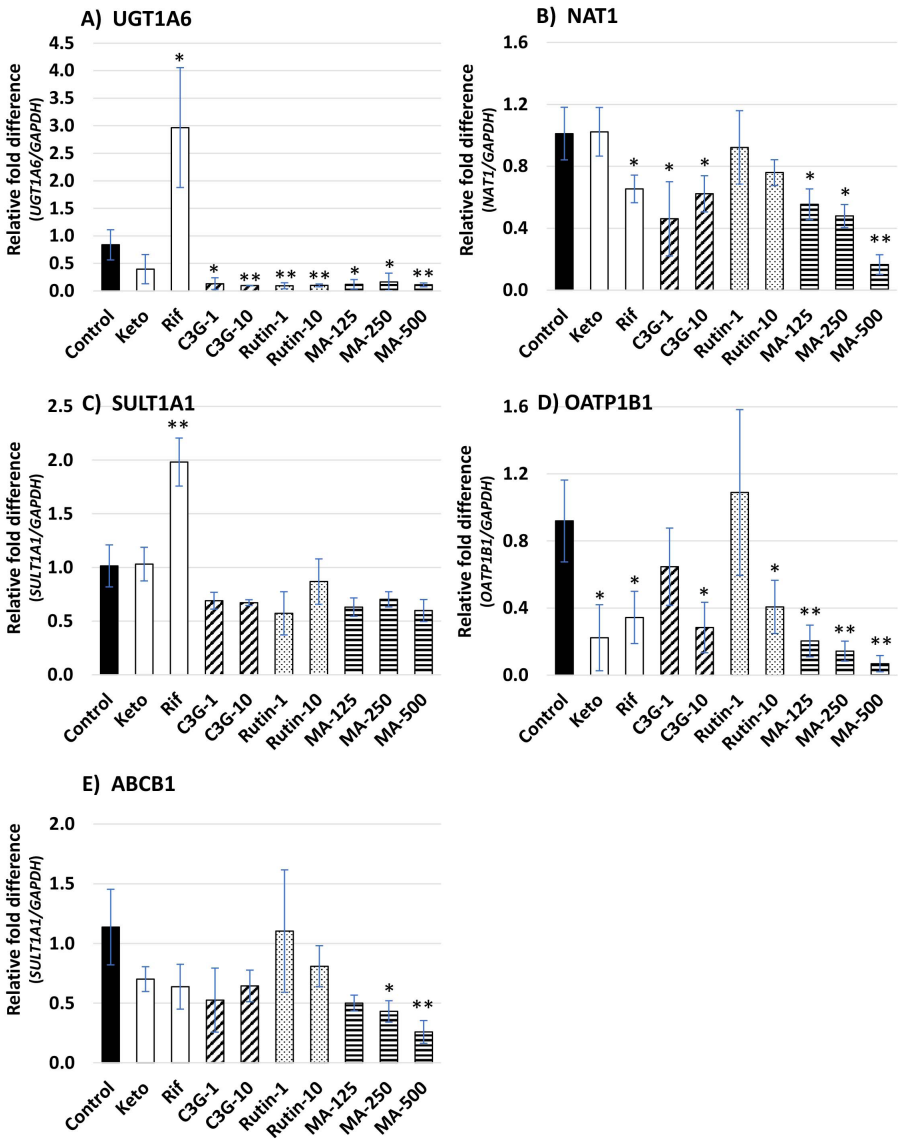

Figure 4. Effects of MA fruit, C3G, and rutin on phase II metabolizing enzymes and transporters mRNA expression in Caco-2 cells. $^{*}p < 0.05$, $^{**}p < 0.001$ versus control (DMSO). Keto, 10 µM ketoconazole; Rif, 10 µM rifampicin; C3G, 1 and 10 µM C3G; Rutin, 1 and 10 µM rutin; MA, 125, 250, and 500 µg/ml MA fruit ($n = 4–5$).

(Fig. 5A and B). Although MA also induced *CYP1A2* on its own, it had the opposite effect on *CYP2C19*, inhibiting the expression (Fig. 3B). The expression of *CYP2D6* mRNA was not altered by all treatments, either as single compounds or in combination with MA (Fig. 5C). APAP and ASA did not change the expression of *CYP3A4* mRNA (Fig. 5D) either on their own or in combination with MA. The treatment of Caco-2 cells with Caf both alone and in combination with MA suppressed the expression of *CYP3A4* mRNA to similar levels, while the combination of Sim with the highest dose of MA (500 µg/ml) suppressed the expression of *CYP3A4* mRNA (Fig. 5D). In a previous study, the Chinese traditional medicine formulation BuChang NaoXinTong, which contains MA twig and some other herbs, was shown to induce the expression of *CYP2C19* in human primary hepatocytes and HepG2 cells via activation of PXR (Sun *et al*., 2016). Similarly, an aqueous extract of mulberry leaves increased *CYP3A4* activity, while an ethanol extract of mulberry leaves reduced *CYP1A2, CYP2D6*, and *CYP2C19* activity in a rat model (Sheng *et al*., 2021). Therefore, there are several other factors such as the part of the MA plant being used, the extraction method employed, and the combination of MA with other compounds and plants that could affect the regulatory profiles of CYP expression.

Effect of combinations of MA fruit and common drugs on phase II conjugation enzymes and transporters

UGT1A6 is an important member of the human *UGT1* family expressed in the liver and extrahepatic tissues, including the intestine, that biotransforms planar phenols and arylamines

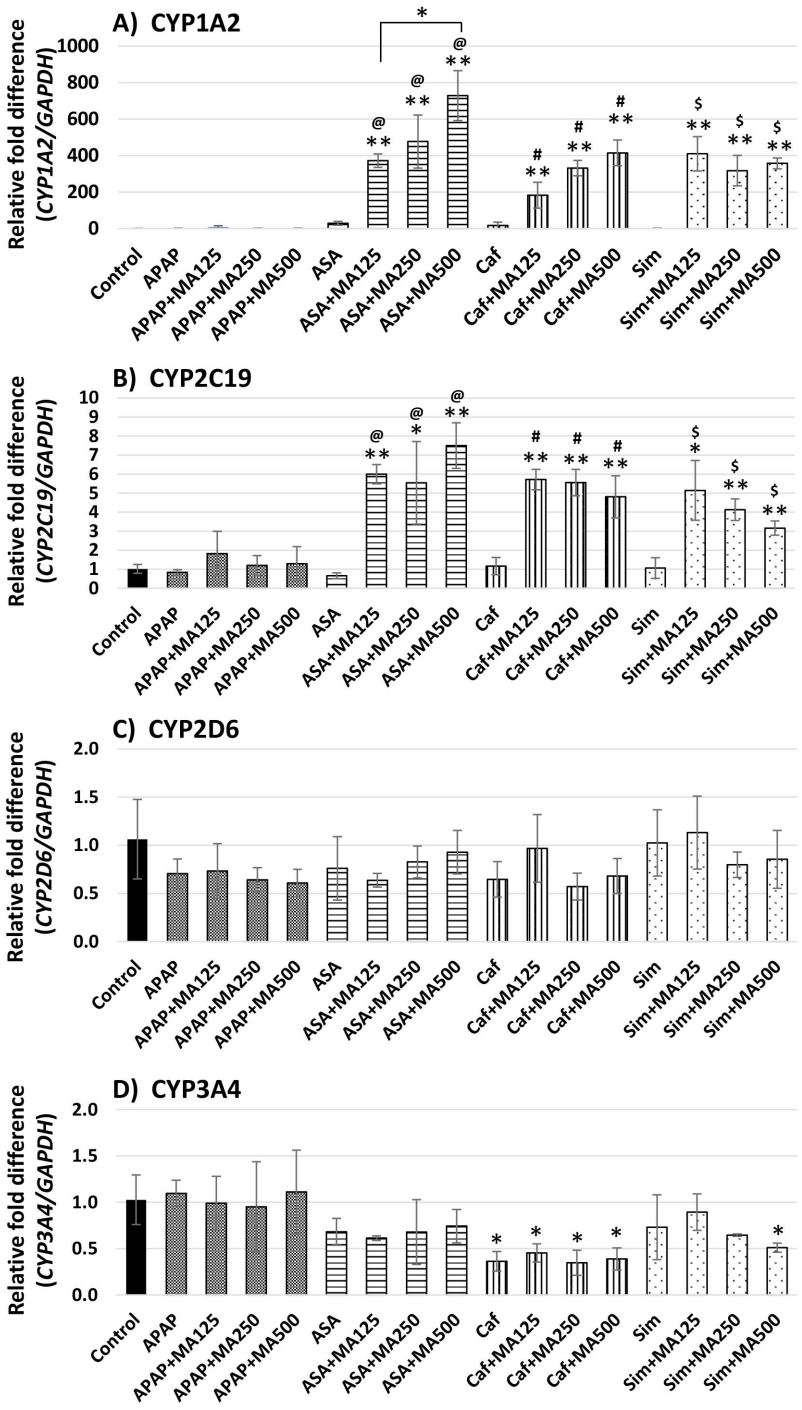

Figure 5. Combinatorial effects of MA fruit and common drugs on CYPs in Caco-2 cells. $^{*}p < 0.05$, $^{**}p < 0.001$ versus control (DMSO); $^{@}p < 0.05$ versus ASA; $^{\#}p < 0.05$ versus Caf; $^{\$}p < 0.05$ versus Sim. APAP, 5 mM paracetamol; ASA, 5 mM aspirin; Caf, 50 µM caffeine; Sim, 10 µM simvastatin; MA, 125, 250, and 500 µg/ml MA fruit ($n = 4–5$).

(Walter Bock and Köhle, 2005). It is responsible for APAP-glucuronide conjugation (Zhao and Pickering, 2011). When tested on their own, both APAP and ASA induced the expression of *UGT1A6* mRNA (Fig. 6A) while MA suppressed *UGT1A6* expression (Fig. 4A). In combination, MA and APAP showed increased induction of *UGT1A6* expression (Fig. 6A). On the other hand, the combination of MA with ASA, Caf, and Sim resulted in the inhibition of the expression of *UGT1A6*. At present, there is limited information about the effect of MA on UGTs. One report found that morusin, a flavonoid isolated from the root bark of MA, inhibited UGTs in liver microsomes of humans, rats, dogs, monkeys, and minipigs (Shi *et al*., 2016).

NAT1 is responsible for the metabolism of pharmacological agents and environmental toxicants

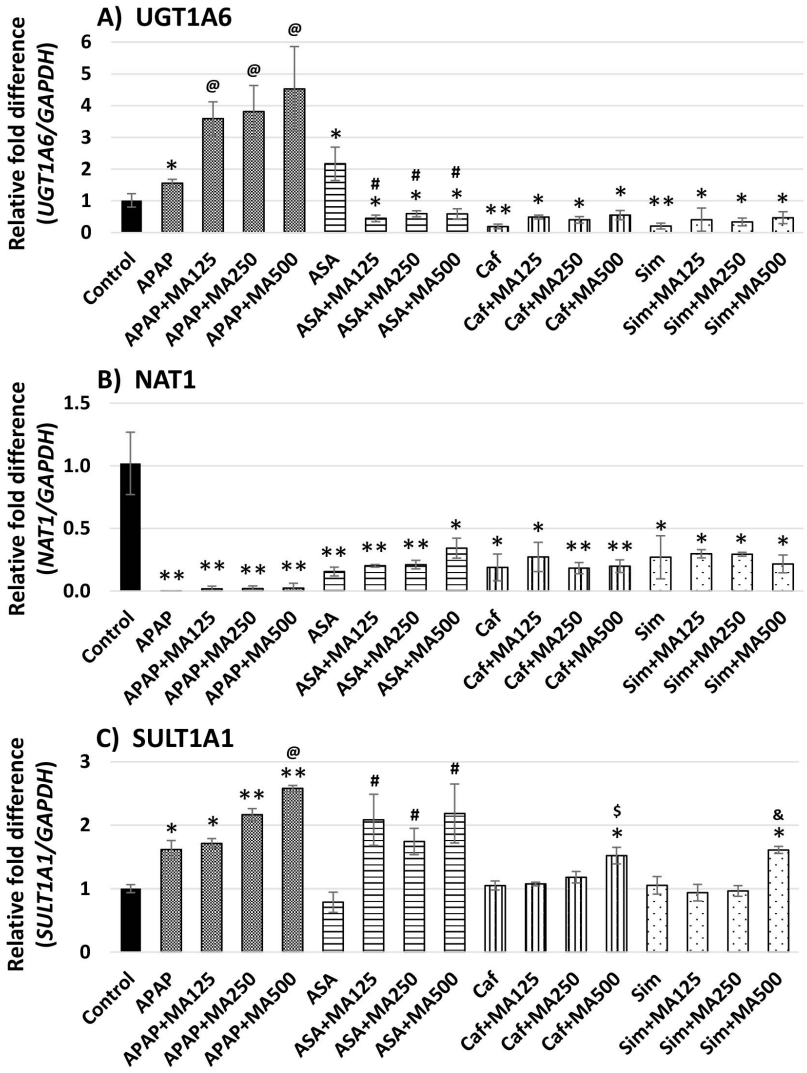

Figure 6. Combinatorial effects of MA fruit and common drugs on phase II metabolizing enzymes in Caco-2 cells. $^{*}p < 0.05$, $^{**}p < 0.001$ versus control (DMSO); $^{@}p < 0.05$ versus APAP; $^{\#}p < 0.05$ versus ASA; $^{\$}p < 0.05$ versus Caf; $^{\&}p < 0.05$ versus Sim. APAP, 5 mM paracetamol; ASA, 5 mM aspirin; Caf, 50 µM caffeine; Sim, 10 µM simvastatin; MA, 125, 250, and 500 µg/ml MA fruit ($n = 4–5$).

(Walker *et al*., 2009). All treatments, including singly applied APAP, ASA, Caf, and Sim and their combinations with MA, extensively suppressed expression of *NAT1* (Fig. 6B).

SULT1A1 is responsible for the sulfation of APAP (Rasool *et al*., 2019). APAP significantly elevated the expression of *SULT1A1* mRNA (Fig. 6C), while ASA, Caf, and Sim did not. Interestingly, ASA, Caf, and Sim in combination with MA significantly induced *SULT1A1* expression.

OATP1B1 transports clinical drugs into cells (Zhang *et al*., 2007). Flavonoids have been reported to inhibit the uptake of *OATP1B1* substrates (Wang *et al*., 2005). APAP, ASA, Caf, Sim, APAP+MA, and ASA+MA all markedly suppressed *OATP1B1* expression, while Caf+MA and Sim+MA controversially showed synergistic induction of *OATP1B1* expression (Fig. 7A). The ethanol extract of MA root was reported to downregulate *ABCB1* expression in MCF7 cells (Choi *et al*., 2013).

ABCB1 is involved in the intestinal absorption of aspirin (Li *et al*., 2017). ASA significantly suppressed the expression of *ABCB1* mRNA (Fig. 7B). However, ASA in combination with the highest dose of MA strongly induced the expression of *ABCB1* mRNA in Caco-2 cells. To date, information about the effect of MA on drug transporters is very limited. It would be interesting to unravel the effect of MA and its constituents on the expression and activity of other drug transporters.

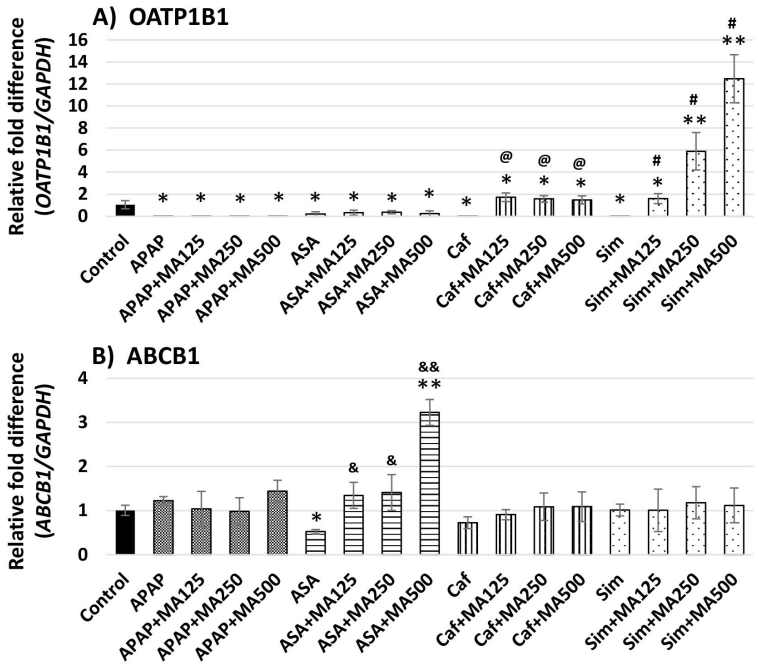

Figure 7. Combinatorial effects of MA fruit and common drugs on drug transporters in Caco-2 cells. $^{*}p < 0.05$, $^{**}p < 0.001$ versus control (DMSO); $^{\&}p < 0.05$, $^{\&\&}p < 0.001$ versus ASA; $^{@}p < 0.05$ versus Caf; $^{\#}p < 0.05$ versus Sim. APAP, 5 mM paracetamol; ASA, 5 mM aspirin; Caf, 50 µM caffeine; Sim, 10 µM simvastatin; MA, 125, 250, and 500 µg/m MA fruit (n = 4–5).

CONCLUSION

MA fruit, C3G, and rutin did not alter the expression of *CYP2D6* and *SULT1A1* in Caco-2 cells. Nevertheless, they down-regulated the expression of *CYP2C19*, *CYP3A4*, *UGT1A6*, *NAT1,* and *OATP1B1*, and MA upregulated the expression of *CYP1A2*. The combination of MA with aspirin, caffeine, or simvastatin showed additional induction of *CYP1A2* and *CYP2C19*, while the combination of MA and paracetamol synergistically upregulated the expression of *UGT1A6* and *SULT1A1*. Therefore, the consumption of MA fruit or mulberry supplements poses a risk for drug interactions via the modulation of CYP and conjugation enzyme-associated metabolism and *OATP1B1*- or *ABCB1*-mediated drug transport in the human intestine.

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CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included within this research article.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit it to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be authors as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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